D8

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 39/00, C12P 21/06
C12N 5/00, 1/00, 15/00
C07K 3/00, 13/00, 15/00
C07K 17/00

A1

(11) International Publication Number: WO 94/09817

(43) International Publication Date: 11 May 1994 (11.05.94)

(21) International Application Number: PCT/US92/09347

(22) International Filing Date: 4 November 1992 (04.11.92)

(71) Applicant (for all designated States except US): CITY OF HOPE [US/US]; 1500 East Duarte Road, Duarte, CA 91010-0269 (US).

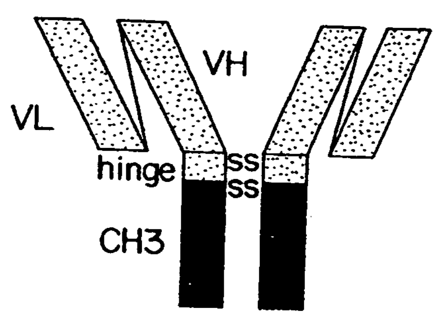
(72) Inventor; and
(75) Inventor/Applicant (for US only) : WU, Anna, M. [US/US]; 14919 Sutton Street, Sherman Oaks, CA 91403 (US).

(74) Agent: IRONS, Edward, S.; 919 18th Street, N.W., Suite 800, Washington, DC 20006 (US).

(81) Designated States: CA, DE, GB, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: NOVEL ANTIBODY CONSTRUCT



(57) Abstract

An antigen binding protein construct or "minibody" which includes the VL and VH domains of a native antibody fused to the hinge region and CH3 domain of the immunoglobulin molecule is described. Minibodies are small versions of whole antibodies which encode in a single chain the essential elements of a whole antibody. Minibodies are expressed by host cells transformed with minibody genes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

NOVEL ANTIBODY CONSTRUCT

This invention was made with government support under Grant Nos. P01CA43904 and R01CA42329 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF INVENTION

This invention relates to a novel antigen binding protein construct or "minibody" which includes the essential elements of an antibody.

BACKGROUND OF THE INVENTION

Previous workers (1)[1/] have demonstrated that single chain antigen-binding proteins can be generated by linking the heavy chain variable domain and light chain variable domain of an antibody into a single protein, using a short linker peptide. The length and composition of the linker can vary (2). Single chain antibodies can be produced in E. coli which retain antigen-binding activity. Milenic (3) provides one recent example of a single chain antibody that binds a tumor antigen. Several other single chain antibodies generated that bind to various haptens and antigens have been reported.

Gillies and Wesolowski (4) demonstrated that the CH2 domain of antibodies can be deleted with retention of the antigen binding function. The CH3 domains of such ΔCH2 antibodies permit dimerization. ΔCH2 antibodies are useful for in vivo diagnostics and potentially therapy (5).

To produce bispecific antibodies, Kostelny et al (6) fused Fab fragments of antibodies to the leucine zipper portions of fos and jun proteins in the

1/ Reference citations appear in the bibliography.

absence of a single chain construct for the antigen combining region. Pack and Pluckthun (7), fused a single chain antibody to amphipathic helices from a four helix bundle or from leucine zipper proteins.

SUMMARY OF THE INVENTION

Minibodies are engineered antibody constructs comprised of the variable heavy (VH) and variable light (VL) chain domains of a native antibody fused to the hinge region and to the CH3 domain of the immunoglobulin molecule. Minibodies are thus small versions of whole antibodies encoded in a single protein chain which retain the antigen binding region, the CH3 domain to permit assembly into a bivalent molecule and the antibody hinge to accommodate dimerization by disulfide linkages. In contrast, native antibodies are comprised of four chains, two heavy and two light.

The size, valency and affinity of the minibody is particularly suited for in vivo targeting. Expression in bacterial or mammalian cells is simplified because minibodies are in single chains.

DESCRIPTION OF THE FIGURES

Figure 1 depicts one form of a minibody in which VL indicates the variable light chain domain and VH indicates the variable heavy chain domain of a native T84.66 antibody. The hinge and CH3 domain are from human IgG.

Figure 2 depicts a dimerized minibody.

Figure 3 depicts the construction of a minibody gene.

Figure 4 depicts CEA binding ability of the expression of the product of a T84.66 minibody.

DETAILED DESCRIPTION OF THE INVENTION

A minibody is a chimeric molecule, typically illustrated by Figure 1, constructed by fusion of a single chain antibody to the hinge region of an

antibody followed directly by the CH3 domain of the immunoglobulin molecule. Two minibodies may dimerize through disulfide linkages as shown by Figure 2.

The Single Chain Antibody

Single chain antibodies are prepared in the manner described by Bird (1), Pantoliano (2), Milenic (3), or Takkinen (8).

EXAMPLE I

A. The Single Chain T84.66 Antibody

This Example demonstrates the production of a single chain construct derived from the T84.66 antibody depicted by Figure 3A in Neumaier, M., et al. Cancer Research 50:2128-2134 (1990) (10).

The T84.66 antibody domains were assembled in order VL-peptide linker-VH. The 14 amino acid peptide linker was identical to the "212 linker" described in Pantoliano et al. Biochemistry 30:10117 (1991) (2).

The construction was accomplished by polymerase chain reaction/splice overlap extension (PCR/SOE). See Horton (9). The heavy and light chain gene segments from T84.66 were amplified separately using long overlapping primers that encoded the desired 14 amino acid linker. In addition, bacterial pelB peptide was added upstream from VL. These regions were overlapped and the entire construct was fused by amplification in a secondary PCR reaction. The primer used for amplifying the C-terminus of VH included an XhoI restriction site to accommodate assembly of the minibody. See Figure 3A.

The single chain construct was transferred into the plasmid pUC18 for DNA sequence analysis, and thereafter into the bacterial expression vector pKKtac (see Takkinen (8)). The expression vector was transfected into E. coli RV308 for expression.

Cultures were grown in rich medium at 37° to an O.D.$_{600}$ of 0.8. Isopropyl thiogalactoside (IPTG) was added to 1 mM to induce synthesis of the single chain antibody. Cultures were then shifted to lower temperature (25° or 30°), and synthesis was allowed to proceed for five hours or overnight.

Because the expression vector includes the pelB signal sequence, the single chain antibody is processed and secreted into the supernatant. CEA-binding activity was detected in the supernatant using a competition ELISA, with murine T84.66 as a standard. Under the best conditions (synthesis at 25°, overnight incubation), CEA-binding activities of 2-5 μg/ml were routinely achieved.

Either affinity chromatography, or a combination of ammonium sulfate precipitation, ion-exchange chromatography, and size fractionation yields pure single chain antibody. A 28K band is seen on silver stained SDS-polyacrylamide gels, and the appropriate size peak is also detected by electrospray mass spectrometry. CEA-binding activity copurifies with the 28K species.

B. Design and Assembly of the Hinge and CH3 Minibody Components

In addition to the single chain antibody, the minibody construct includes the hinge and CH3 domains from an immunoglobulin molecule. Examination of the 3-D structure of the human IgG 1 antibody indicates that the native hinge peptide may be too short to span the distance between the N-termini of the CH3 domains, when the CH2 domain has been deleted. Accordingly, a ten amino acid sequence of glycine and serine residues was added to the hinge and CH3. See Figure 3B.

Assembly of the human IgG 1 hinge region and CH3 domains were accomplished by the polymerase chain reaction/splice overlap extension method. See Horton (9). The overlapping primers used to generate the fusion also encoded the additional ten amino acid glycine/serine linker. Furthermore, the flanking primers incorporated an Xho I site on the 5′ end and a Hind III site on the 3′ end of the hinge-CH3 segment. See Figure 3B. These restriction sites were added to facilitate cloning and construction of the minibody. The hinge-CH3 construct was subcloned into pUC18, and DNA sequence analysis confirmed the correct sequence had been assembled.

C. The Final T84.66 Minibody Construct

The single chain minibody (including the pelB leader region) and the hinge-CH3 components were isolated from their separate pUC18 plasmids as Eco RI-Xho I and Xho I-Hind III restriction fragments, respectively. The Xho I sites were ligated, and the resulting Eco RI-Hind III minibody gene was recloned into pUC18 to provide a T84.66 minibody. See Figure 3C. Correct assembly was verified by restriction digestion and DNA sequence analysis.

EXAMPLE II

Expression of the T84.66 Minibody

The entire T84.66 minibody gene construct described in Example I was transferred as an Eco RI-Hind III restriction fragment into the bacterial expression vector pKKtac (8) as described above. The resulting plasmid was transfected into *E. coli* RV308 for minibody expression.

Pregrowth and induction with IPTG carried out as described in Example IA. Following induction, protein synthesis was allowed to occur at 26°C for 3 to 20 hours. A competition assay was performed

to measure the expression of soluble, secreted minibody in the bacterial supernatants. Titer plate assay wells were coated with CEA, blocked, and sample supernatants were allowed to bind. Murine T84.66 was added as competitor. The extent of competition was determined by ELISA with the result shown by Figure 4.

Figure 4 demonstrates that four independent clones of E. coli RV308 transformed with the minibody expression plasmid (MiAb #s 11, 12, 31 and 32) as well as the single chain antibody (SCA212) yield an expression product having CEA-binding activity. Activities reached >2μg/ml in the culture supernatants in this experiment. Control cultures (pKK) were negative.

Three lines of evidence indicate that the minibody described in Example II assembles into dimers.

First, a valency greater than one is indicated by an ELISA valency assay. Assay plates were coated with CEA, the minibody sample is added and allowed to bind to the CEA, and the plates are washed. The immobilized minibody was then incubated with biotinylated-CEA. If the minibody is bivalent, it can capture the second CEA molecule and score positive in a color reaction, which it did. Thus, the minibody is at least bivalent.

Second, a native gel shows that the cultures induced to secrete minibody produce a 80-90 K protein in the culture supernatant. This protein is apparently the T84.66 minibody.

Third, gel-filtration high-performance liquid chromatography (HPLC) indicates that the CEA-binding activity of the minibody elutes as a single peak, and the apparent molecular weight is between the IgG (150K) and albumin (68K) standards.

The minibody dimer has an appropriate molecular weight for in vivo imaging and therapy studies, it is bivalent for higher affinity, and it demonstrates the feasibility of bispecific minibodies which concurrently bind two different ligands.

BIBLIOGRAPHY

1. Bird et al. Science 242:423 (1988)
2. Pantoliano et al. Biochemistry 30:10117 (1991)
3. Milenic et al. Cancer Research 51:6363 (1991)
4. Gillies and Wesolowski, Human Antibodies and Hybridomas 1:47 (1990)
5. Mueller et al. Proc. Nat. Acad. Sci. 87:5702 (1990)
6. Kostelny et al, J. Immunology 148:1547 (1992)
7. Pack and Pluckthun, Abstract presented at Second Annual IBC International Conference on Antibody Engineering, December 16-18, 1991, San Diego
8. Takkinen, et al. Protein Engineering 4:837-841 (1991)
9. Horton, et al. Gene 77:61-65 (1989)
10. Neumaier, M., et al. Cancer Research 50:2128-2134 (1990)

CLAIMS:

1. A minibody as depicted by Figure 1.

2. A minibody dimer as depicted by Figure 2.

3. A chimeric construct consisting essentially of the light and heavy chain variable domains of a native antibody fused to hinge and CH3 domains of an immunoglobulin molecule.

4. A minibody consisting essentially of the light and heavy chain variable domains of the T84.66 CEA antibody fused to the hinge and CH3 domains of human IgG.

5. The T84.66 minibody depicted by Figure 3C.

6. E. coli RV308 transformed with a plasmid having an expression vector of a minibody as defined by claim 4.

7. A method for constructing a minibody which comprises:

(i) providing a single chain antibody including the variable light chain and variable heavy chain domains of a native antibody, and

(ii) fusing said single chain antibody to a construct of a native antibody hinge region and CH3 domain.

8. A method as defined by claim 7 in which said native antibody is a CEA antibody.

9. A method as defined by claim 7 in which said native antibody is T84.66 CEA antibody.

10. A method as defined by claim 7 in which said antibody is T84.66 antibody to CEA and in which said hinge region and CH3 domain are the human IgG hinge region CH3 domain.

11. The single chain T84.66 CEA antibody depicted by Figure 3A.

12. A bispecific minibody dimer, in which the variable light (VL) domain and variable heavy (VH) domain are derived from different antibodies.

13. A method of producing a minibody which comprises transforming a host cell with the nucleic acid sequence of a minibody gene and culturing the transformed host cell under conditions which permit expression of said nucleic acid sequence by said host cell.

14. A method of producing a T84.66 minibody as depicted by Figure 3C which comprises transforming a host cell with the nucleic acid sequence of said minibody and culturing the transformed host cell under conditions which permit expression of said nucleic acid sequence by the host cell.

15. A method as defined by claim 14 in which said host cell is E. coli.

16. A minibody produced by the method of claim 13 or claim 14.

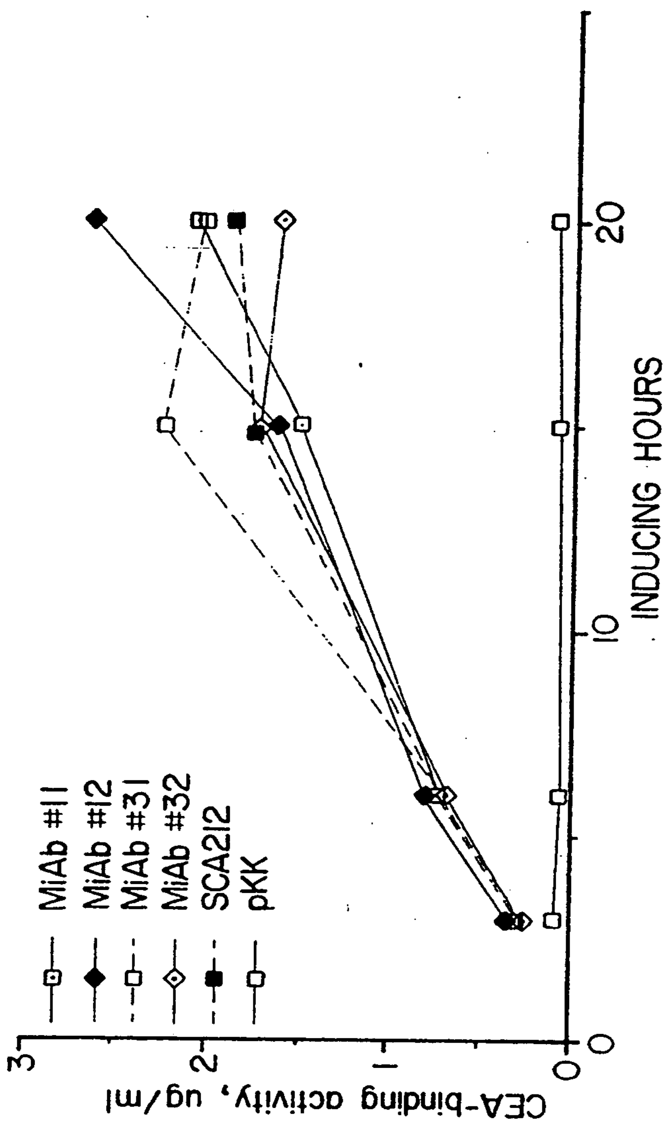

MiAb #11
MiAb #12
MiAb #31
MiAb #32
SCA212
pKK
CEA-binding activity, ug/ml
0
1
2
3
0
10
20
INDUCING HOURS

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09347

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :424/85.8; 435/69.6, 172.3, 240.27, 320.1, 849, 972; 530/388.5, 867
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 435/69.6, 172.3, 240.27, 320.1, 849, 972; 530/388.5, 867

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS FILE:USPAT; DIALOG FILES:5,55,155,399,350,351,73
KEYWORDS: MINIBOD?, ANTIBOD? OR IMMUNOGLOBUL? OR IG? ?, AND SINGLE(W)CHAIN, DELET? OR MUTAT? OR ALTER?, CH2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CANCER RESEARCH, VOLUME 51, ISSUED 01 DECEMBER 1991, MILENIC ET AL, "CONSTRUCTION, BINDING PROPERTIES, METABOLISM, AND TUMOR TARGETING OF A SINGLE-CHAIN F_v DERIVED FROM THE PANCARCINOMA MONOCLONAL ANTIBODY CC49", PAGES 6363-6371, SEE ENTIRE DOCUMENT.	1,7,8 3-6,9-11, 13-16
Y	PRODEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOLUME 81, ISSUED NOVEMBER 1984, MORRISON ET AL, "CHIMERIC HUMAN ANTIBODY MOLECULES: MOUSE ANTIGEN-BINDING DOMAINS WITH HUMAN CONSTANT REGION DOMAINS", PAGES 6851-6855, SEE ENTIRE DOCUMENT.	1-16
Y	SCIENCE, VOLUME 242, ISSUED 21 OCTOBER 1988, BIRD ET AL, "SINGLE-CHAIN ANTIGEN-BINDING PROTEINS", PAGES 423-426, SEE ENTIRE DOCUMENT.	1-16
Y	HUMAN ANTIBODIES AND HYBRIDOMAS, VOLUME 1, NO. 1, ISSUED 1990, GILLIES ET AL, "ANTIGEN BINDING AND BIOLOGICAL ACTIVITIES OF ENGINEERED MUTANT CHIMERIC ANTIBODIES WITH HUMAN TUMOR SPECIFICITIES", PAGES 47-54, SEE ENTIRE DOCUMENT.	1-16
Y	CANCER RESEARCH, VOL. 50, ISSUED 01 APRIL 1990, NEUMAIER ET AL, "CLONING OF THE GENES FOR T84.66, AND ANTIBODY... HAMPSTER OVARY CELLS", PAGES 2128-2134, ENTIRE DOCUMENT.	1-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 DECEMBER 1992	26 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer T. MICHAEL NISBET Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/09347

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 39/00; C12P 21/06; C12N 5/00,1/00,15/00; C07K 3/00,13/00,15/00,17/00